New Generation Vaccines

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New and Improved Vaccines Against Cholera

Part i: Attenuated *Vibrio cholerae* O1 and O139 Strains as Live Oral Cholera Vaccines

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I. INTRODUCTION

An ideal cholera vaccine should possess a number of characteristics regarding safety, efficacy and practicality. In our view, an ideal cholera vaccine would (1) be well tolerated in all age groups; (2) protect high-risk groups (including young children and persons of blood group O who had not been well protected in earlier trials [1–3]; (3) require only a single dose; (4) be administered orally for practicality and for better stimulation of the intestinal immune system; (5) stimulate both anticolonizing and antitoxic immunity; (6) confer a high level of protection (>80% efficacy) that would endure for at least 5 years; (7) begin protecting within a few days of administering the single oral dose (important for use in explosive outbreak situations); (8) be available in a simple formulation that would retain potency when stored in tropical climates and would facilitate mass vaccination, including vaccination of young children; and (9) be inexpensive.

Other approaches to cholera vaccines, such as oral killed whole-cell/B subunit (reviewed in Chaper 33ii) and parenteral O polysaccharide–protein conjugates (reviewed in Chapter 52) do not meet one or more of the above requirements. We think that live, attenuated *Vibrio cholerae* vaccines strains administered orally offer the best possibility for meeting all of the above characteristics.

The rationale for the use of attenuated *V. cholerae* strains as live oral cholera vaccines is based on several observations that have been extensively reviewed elsewhere [3–7]:

1. A single clinical infection due to wild-type *V. cholerae* O1 confers significant protection against cholera upon subsequent exposure to wild-type *V. cholerae* O1.
2. Although many virulence properties contribute to the pathogenesis of cholera, the in vivo expression of cholera enterotoxin is a prerequisite for the profuse purging of voluminous rice-water stools that is characteristic of cholera gravis.
3. The critical protective immunity to cholera is antibacterial rather than antitoxic in nature, although in the short term, antitoxic immunity may synergistically enhance antibacterial immunity.
4. The degree of stimulation of serum vibriocidal antibody following ingestion of a live oral cholera vaccine or following infection with wild-type *V. cholerae* O1 constitutes the best correlate of antibacterial immunity in the intestine.
5. The critical antigen or combination of antigens that provide protective immunity in humans has not been definitively identified.

The development of live, attenuated vaccines to prevent cholera has been greatly aided by the application of recombinant DNA technology. The major virulence factor of *V. cholerae* is cholera enterotoxin, which is responsible for the large volumes of watery diarrhea that is the hallmark of this disease [4,6]. Before the development of recombinant DNA technology, many attempts were made to attenuate *V. cholerae* O1 using chemical mutagenesis to derive a strain that produced the nontoxic yet immunogenic B subunit of the cholera toxin but not the A subunit, which is the enzymatically active portion of the toxin. It is now known that most strains of *V. cholerae* O1 contain multiple copies of the *ctxAB* genes, which encode cholera toxin, thereby making the development of a immunogenic yet nontoxic strain nearly impossible by chemical mutagenesis [8]. One attenuated strain of *V. cholerae* O1 El Tor containing a single *ctx* gene copy was successfully mutated by chemical treatment to express the B but not the A subunit of cholera toxin [9]. However, this strain, Texas Star-SR, proved to be unexpectedly reactogenic in volunteers [10]. Because of this reactogenicity, the then-unknown nature of the genetic lesion, and the development of recombinant DNA technology and its promise for the development of new attenuated strains, Texas Star-SR was not further investigated. Nevertheless, this strain proved the principle that an attenuated *V. cholerae* strain could provide protection against experimental challenge with virulent *V. cholerae* and served as the foundation for the recombinant *V. cholerae* vaccine strains that were to follow.

II. EARLY GENERATIONS OF RECOMBINANT ATTENUATED CHOLERA VACCINE CANDIDATES

The basic method involved in developing live recombinant vaccines against cholera has been to clone and sequence genes encoding specific virulence factors, mutate these genes in vitro using recombinant techniques, and then reintroduce the mutated genes into wild-type *V. cholerae* via homologous recombination. The crucial common mutation in the early and later generations of attenuated cholera vaccines is the deletion of genetic sequences encoding the *ctxA* gene encoding the cholera enterotoxin A subunit, which is responsible for the ADP-ribosylating activity of the holotoxin. Rather than chemically mutagenized strains in which only one nucleotide in the *ctxA* gene is mutated, the construction of the recombinant vaccine candidates involved the deletion of several hundred nucleotides, thereby preventing the reversion of the attenuated strain to wild-type toxigenicity. A number of such vaccines have been constructed by investigators at the Center for Vaccine Development at the University of Maryland [11–15] and at Harvard University [16–20].

The first-generation recombinant cholera vaccines were generated from wild-type El Tor strain N16961 and classical strain 395. These vaccine candidates, JBK70 [13] from N16961 and CVD 101 [12] and 395N1 [16] from 395, were markedly attenuated compared to the wild parent strain and were highly immunogenic. When volunteers who were immunized with a single dose of 10^6 JBK70 were challenged with the virulent parent strain, significant protection was observed [21]. Diarrhea occurred in 7 of 8 unimmunized controls but in only 1 of 10 vaccinees, for a vaccine efficacy of 89%. This level of efficacy was equivalent to that seen following sequential experimental infections with wild-type El Tor strains. Interestingly, JBK70 produced neither the A nor B subunits of cholera toxin, so this challenge study demonstrated the importance of antibacterial immunity in the absence of antitoxic immunity.

Despite the high levels of immunity engendered by these three vaccine candidates, they were unexpectedly reactogenic. Approximately one-half of the recipients suffered adverse reactions such as mild diarrhea, malaise, nausea, vomiting, abdominal cramps, low-grade fever, and headache [21,22]. These strains never caused severe or even moderate diarrhea but were nonetheless not studied further because of these reactions.

In addition to the knowledge gained from clinical trials with these strains, two other attenuated strains derived from 395N1 gave valuable information regarding the pathogenicity and immunogenicity of *V. cholerae*. TCP2 is a further derivative of 395N1 that is mutated in *tcpA*, the gene encoding the structural subunit of the toxin coregulated pilus (TCP), and JJM43 is mutated in the gene encoding ToxR, which regulates both *tcpA* and *ctxAB*. Both strains were markedly diminished in intestinal colonization ability and in immunogenicity, demonstrating the importance of retaining both of these factors in attenuated cholera vaccines [22].

III. RECOMBINANT ATTENUATED *VIBRIO CHOLERAE* O1 STRAIN CVD 103-HgR

When the first attenuated *V. cholerae* strains lacking only *ctxA* were found to cause mild to moderate di-

arrhea in volunteers, two hypotheses were proposed to explain this response [21]. The first hypothesis was that a previously unknown enterotoxin was responsible for the diarrhea in the absence of cholera enterotoxin. The second hypothesis was that avid colonization by the attenuated *V. cholerae* strain in the proximal small bowel, a site where only low numbers of bacteria are found in normal North Americans, would somehow cause diarrhea and other symptoms. Investigations proceeded at the Center for Vaccine Development along both avenues to develop further attenuated cholera vaccine strains.

The first recombinant vaccine strain to be well tolerated yet highly immunogenic and protective was strain CVD 103, derived from the classical Inaba strain 569B. This parent strain was reported to lack a Shiga-like toxin that was proposed to be involved in the vaccine reactogenicity [23]. In addition, this parent strain also colonized the intestine to lower levels than other toxigenic *V. cholerae* strains. CVD 103 was derived from 569B by deletion of the *ctxA* gene [14]. A single dose of CVD 103 vaccine elicited seroconversions of vibriocidal antibody in 90% of vaccinees and of antitoxin in >80% [24]. When vaccinees were challenged with toxigenic *V. cholerae* O1 strains, significant protection was seen against strains of either serotype or biotype.

Before CVD 103 could be tested in outpatient studies, where it would likely be shed into the environment, genes encoding resistance to mercury (*mer*) were added to provide a marker to readily differentiate the vaccine strain from wild-type *V. cholerae*. The *mer* genes were recombined into the chromosomal *hlyA* locus. The *hlyA* gene encodes a hemolysin and previous studies had shown that this gene could be mutated without affecting colonization, immunogenicity or reactogenicity [21]. The resulting strain, CVD 103-HgR, exhibits many of the characteristics of an ideal cholera vaccine and has been extensively studied in numerous countries.

A. Safety of CVD 103-HgR

To date, more than six thousand subjects ranging in age from 7 months to 65 years have participated in placebo-controlled clinical trials carried out in industrialized countries (the United States, Switzerland, Italy) [5,24,–28] and in developing countries with endemic cholera (Indonesia, Thailand) [29–32], epidemic cholera (Peru, Colombia, Mexico) [33,34], or little or no cholera (Chile, Costa Rica) [35,36]. In all studies, neither diarrhea or any other adverse reaction occurred significantly more often in vaccinees than in placebo recipients.

B. Immun genicity

The serum vibriocidal response is the best correlate of protection for *V. cholerae* O1 and the best measure of successful stimulation of antibacterial immunity [3–6,37]. The serum antibodies are believed to serve as a marker for the elicitation of protective intestinal immune responses. In the immunogenicity trials of CVD 103-HgR and other attenuated *V. cholerae* O1 vaccine candidates, a fourfold or greater rise is considered significant (i.e., seroconversion). In clinical trials in North American, Swiss, and Italian volunteers, a single 5×10^8 colony-forming unit (CFU) dose has engendered fourfold or greater rises in vibriocidal antibodies in approximately 90% of vaccinees [5,25,27].

In the initial immunogenicity trials in developed countries, volunteers received a dose of 5×10^8 CFU, which was highly immunogenic in this population. However, in initial trials involving Thai soldiers and Indonesian children, only 25 and 16%, respectively, of the subjects fed this dosage seroconverted [30,31]. It was subsequently found that many individuals in endemic areas already have elevated vibriocidal titers and are presumably at least partially immune [30,31]. In several studies, the baseline vibriocidal geometric mean titer (GMT) in subjects who did not seroconvert was significantly higher than the baseline GMT of individuals who did seroconvert [30,31]. In such persons with elevated baseline titers, a further rise in serum vibriocidal antibody is not a good measure of vaccine "take" or boosting. When the dose of vaccine was increased to 5×10^9 CFU for use in developing countries, a marked increase in seroconversion rate was seen in these populations, with a single dose eliciting seroconversions in 75–85% of subjects [30–35]. The seroconversion rate in subjects of blood group O is important, since in the large field trial of two inactivated oral cholera vaccines in Bangladesh, persons of blood group O were less well protected than persons of non-O blood groups [2,3]. The 5×10^9 CFU dose of CVD 103-HgR caused equal seroconversion rates among persons of blood groups O and non-O in Santiago, Chile; interestingly, the GMT of vibriocidal antibody was significantly higher among subjects of blood group O than among those of other blood groups [35].

C. Efficacy in Volunteer Challenge Studies

The ability of CVD 103-HgR to protect against cholera has been studied in the volunteer model of experimental cholera at the University of Maryland. A total of 10 such challenge studies have been performed, 3 studies with volunteers who received CVD 103 and 7

studies with volunteers who received CVD 103-HgR [5,24,28,38]. In all but one study volunteers received only a single dose of vaccine; in one study, volunteers received two doses a week apart. In each of these studies, CVD 103-HgR (or its parent CVD 103) conferred significant protection against challenge with fully enterotoxigenic wild-type *V. cholerae* O1. As shown in Table 1, CVD 103 and CVD 103-HgR provided 100% protection against severe (≥5.0-L total purge) and moderate (≥3.0-L total purge) diarrhea. In some vaccinees, mild diarrhea occurred after challenge. The overall protective efficacy against any diarrhea was quite high after challenge with toxigenic strains of the classical biotype (82–100% irrespective of serotype) and moderate after challenge with El Tor strains (49–67% irrespective of serotype). Since it is the syndrome of cholera gravis—leading to dehydration—rather than mild diarrhea that gives cholera significance as a public health problem, complete protection against moderate to severe diarrhea indicates that CVD 103-HgR could be an effective public health tool in vaccination programs.

One potential use of an attenuated live cholera vaccine is in outbreaks in refugee camps or similar situations. To test how rapidly protection is induced by CVD 103-HgR, Tacket et al. [28] challenged volunteers with a toxigenic classical Inaba strain a mere 8 days after they had ingested a single dose of the vaccine. Complete protection was seen in this study, indicating that the onset of protection is quite rapid. Similarly, volunteers were also challenged 6 months after immunization to assess the duration of immunity. Complete protection was again seen 6 months after immunization, the longest interval so far tested.

D. Field Trial of Efficacy

Although the results of challenge studies in North American volunteers and immunogenicity studies in developing countries are highly encouraging, the key test of CVD 103-HgR in controlling cholera will be a large-scale field trial in a country endemic for cholera. In 1993, a randomized, double-blind, placebo-controlled field trial was initiated in about 67,000 subjects 2–42 years of age in North Jakarta, Indonesia, to assess the efficacy of a single oral dose of CVD 103-HgR in protecting against cholera in a population exposed to natural challenge. Surveillance will be maintained until at least fall of 1997 before data are analyzed. This field trial is being conducted by the Indonesian Ministry of Health and the U.S. Naval Medical Research Unit–2 under the sponsorship of the World Health Organization.

Table 1 Efficacy of CVD 103 and 103-HgR Live Oral Cholera Vaccines Against Experimental Challenge with Wild-Type *V. cholerae* O1

Severity of diarrhea[a]	Controls	Vaccinees	Efficacy (%)
≥5.0	9/88	0/99	100
≥3.0	19/88	0/99	100
≥2.0	28/88	2/99	94
≥1.0	42/88	7/99	86
Any diarrhea	70/88	19/99	76

[a]Total diarrheal stool volume in liters during episode of experimental cholera.
Source: Refs. 5, 24, 28, and 38.

E. Formulation

A key consideration for the success of attenuated cholera vaccines is the development of a practical formulation suitable for use in large-scale vaccination programs. For all trials of CVD 103-HgR outside the United States, the vaccine was formulated as two aluminum foil sachets, one containing lyophilized vaccine (and aspartame as sweetener) and the other containing buffer (to protect the vaccine strain from gastric acid). The two sachets are mixed in a cup containing 100 mL of water and the resultant suspension is ingested by the subject. In Chapter 70, Cryz and colleagues describe the manufacturing process involved in large-scale production of this vaccine formulation.

IV. RECOMBINANT ATTENUATED *VIBRIO CHOLERAE* O1 EL TOR VACCINE STRAINS

The attenuated *V. cholerae* CVD 103-HgR vaccine strain belongs to the classical biotype of *V. cholerae* O1. The current predominant O1 biotype throughout the world is the El Tor biotype. As discussed above, this vaccine provided 100% protection against moderate or severe diarrhea when vaccinees were challenged with toxigenic El Tor Inaba or Ogawa strains. However, CVD 103-HgR provided reduced protection against mild diarrhea caused by El Tor challenge strains (49–67%) relative to the protection seen against mild diarrhea caused by classical challenge strains (82–100%). Thus, the addition of an attenuated El Tor vaccine strain to classical CVD 103-HgR may provide some additional protection against mild diarrhea, at least in North American volunteer studies. However, there is compelling evidence, both from ep-

idemiology and volunteer studies, that infection with the classical biotype is a more powerful immunizing experience than infection with the El Tor biotype. Clemens et al. [39] studied cholera in Bangladesh during a time in which both classical and El Tor biotypes were causing disease. Over a 42-month observation period, 2214 initial episodes of cholera were recorded in the study population. Seven of these individuals had a second episode of cholera. An initial infection with a classical strain provided 100% protection against a second episode of cholera due to either classical or El Tor strains. In contrast, an initial infection with El Tor provided only 30% protection against a second El Tor episode and no protection whatsoever against subsequent classical biotype disease. In studying short-term protection in North American volunteers infected with wild-type *V. cholerae* O1 strains, an initial infection with a classical strain led to 100% protection upon subsequent challenge, whereas an initial infection with an El Tor strain led to about 90% protection [5].

The reasons for the differences in protection induced by El Tor and classical strains are unknown. The regulation of *ctx* and *tcp* gene expression and response to environmental conditions are different in the two biotypes. One crucial virulence factor, the TCP colonization factor, shares only 82% protein sequence homology of the major pilin subunit in El Tor and classical strains [40]. Support for the importance of TCP sequence differences in biotype-specific protection comes from animal studies using passive immunization against El Tor or classical TCP preparations [41]. However, infection with either El Tor or classical strains in humans leads to little or no immune response against either form of TCP, although TCP is clearly essential for intestinal colonization and generation of a protective immune response [42]. A mannose-sensitive hemagglutinin (MSHA) expressed by El Tor but not classical strains has been proposed to be important in intestinal colonization and biotype-specific protection [43]. However, recent animal studies using isogenic El Tor strains mutated in the gene encoding MSHA show no involvement of the MSHA in intestinal colonization [44]. Despite the lack of certainty as to the critical differences between El Tor and classical biotypes, a variety of attenuated El Tor vaccine candidates have been prepared using molecular genetic techniques similar to those employed in the construction of classical candidates.

A. CVD 110 and CVD 111

After volunteer trials with the initial reactogenic recombinant *V. cholerae* vaccines, novel *V. cholerae* enterotoxins were discovered, and it was hypothesized that these toxins were responsible for the reactogenicity of these early vaccine strains. Two such toxins, Zot (zonula occludens toxin) and Ace (accessory cholera enterotoxin), were found to have enterotoxic properties in rabbit ileal tissue mounted in Ussing chambers [45,46]. The *zot* and *ace* genes are located immediately upstream of *ctx* in a 4.5-kb region called the "core region" [45–47]. Mekalanos and colleagues have shown that this 4.5-kb region is flanked in El Tor strains by two more copies of a 2.7-kb element called RS1 [47]. This region was recently shown to encode a filamentous bacteriophage [48], and at least the *zot* gene is essential for phage morphogenesis, suggesting that Zot and Ace may have dual functions. To address the hypothesis that the putative Zot and Ace toxins were responsible for the reactogenicity of the attenuated *V. cholerae* O1 strains, Michalski et al. [15] constructed a derivative of El Tor strain E7946 in which the entire 4.5-kb core element containing *ace*, *zot*, and *ctx* (and two other open reading frames) was deleted by homologous recombination between the flanking RS1 elements. To provide expression of the CT B subunit, the *ctxB* gene was cloned under the control of the *ctx* promoter and inserted along with *mer* genes into the *hlyA* gene on the *V. cholerae* chromosome. Although the resulting strain, CVD 110, provided excellent vibriocidal and antitoxic immune responses in volunteers, it caused mild to moderate diarrhea in 7 of 10 vaccinees (which was accompanied by malaise, nausea, and low-grade fever) and was therefore unacceptably reactogenic [49].

The same genetic manipulations used to construct CVD 110 were applied to a different toxigenic El Tor strain, N16117. In previous volunteer studies, this parent strain was not as virulent as other wild-type El Tor challenge strains [7]. The resulting vaccine candidate, CVD 111, lacked *ctxA*, *ace*, and *zot* genes and expressed the CT B subunit and mercury resistance. When tested in volunteers, CVD 111 caused mild diarrhea in 3 of 25 volunteers (12%) at a dose of 10^9 CFU [50]. The seroconversion rate for both vibriocidal and antitoxin responses was 92% after a single dose. Seven weeks after vaccination, 18 vaccinees and 8 unimmunized controls underwent wild-type challenge with El Tor Ogawa strain 3008. Three (16.7%) of 18 vaccinees and 7 (87.5%) of 8 controls developed diarrhea, for a vaccine efficacy of 80.9%. Further studies are under way to determine whether lower doses of CVD 111 are better tolerated yet maintain protection against wild-type El Tor challenge. Phase 2 studies are also under way to evaluate the safety and immunogenicity of a bivalent vaccine consisting of CVD 111 and CVD 103-HgR.

B. Peru-14 and Peru-15

Taylor et al. [19] have reported the development and testing of attenuated El Tor vaccine candidates derived from wild-type El Tor strains from Peru (C6709), Bahrain (E7946), and Bangladesh (P27549). These strains were attenuated by deletion of the 4.5-kb core element, containing *ctx*, *ace*, and *zot*, as well as the RS1 element and *attRS1* sequences flanking the core element. The 18-bp *attRS1* site was previously shown to be the site of insertion for the genetic element containing *ctx* [47]. In addition, the *ctxB* gene was cloned under the control of the *htpG* heat-shock promoter and inserted into the chromosomal *recA* gene, thereby inactivating this gene involved in homologous recombination. The attenuated strains were designated Peru-3, Bah-3, and Bang-3, reflecting the countries from which the parent strains were isolated. When tested in volunteers at doses of 10^6–10^8, significant rises in vibriocidal antibodies occurred in 14 (93%) of 15 volunteers and in antitoxin in 6 of 15 volunteers (40%) [19]. However, these strains caused diarrhea and other symptoms in 6 of 15 subjects (40%).

This group isolated a further spontaneous derivative of Peru-3 that displayed a filamentous phenotype and a motility defect. This strain, called Peru-14, also showed decreased colonization in a suckling mouse model compared to Peru-3. When tested in doses of 10^6 to 10^9, this strain caused diarrhea in 1 (5%) of 21 subjects. In challenge studies, 4 of 5 vaccinees were protected from challenge with toxigenic El Tor [19]. However, the filamentous phenotype of this vaccine caused clumping, which made dosage calculations difficult, and the strain spontaneously reverted to motility with successive passages [20]. Therefore, another spontaneous mutant of Peru-3 was isolated that was nonfilamentous and stably nonmotile called Peru-15 [20]. At a dose of 2×10^8, none of 11 vaccinees developed diarrhea. Of 11 volunteers, 10 exhibited greater than fourfold rises in vibriocidal antibody titers, and 2 of 11 had greater than fourfold rises in antitoxin antibody. When 5 recipients of Peru-15 and 5 unimmunized controls were challenged with toxigenic El Tor, diarrhea was seen in 2 vaccinees (stool volume <1 L) and in 4 controls (mean stool volume of 1.6 L) [20]. Additional clinical trials are needed to furth r characterize the protective efficacy of this well-tolerated vaccine candidate.

V. LIVE ATTENUATED VACCINE CANDIDATES AGAINST *Vibrio cholerae* O139

Before 1992, cholera was exclusively associated with *V. cholerae* of the O1 serogroup. However, in late 1992, epidemic cholera cause by a *V. cholerae* strain of a serogroup other than O1 appeared in India and Bangladesh. The serogroup associated with this new epidemic was designated O139 [51]. By late 1993, the O139 serogroup had largely displace O1 as the principal cause of cholera in Bangladesh and Calcutta, India, although by 1995, O1 had returned as the predominant serogroup in these countries [52,53]. There was a notable age distribution in this new epidemic of O139 cholera. In Bangladesh and other cholera-endemic countries, the attack rates for O1 cholera are highest in young children and decrease in older age groups, indicating acquisition of immunity in adults after repeated antigenic contact. In contrast, the attack rate for the initial O139 outbreak was higher in adults than in children, suggesting that antibacterial and antitoxic immunity engendered by *V. cholerae* O1 did not confer cross-protection against disease due to *V. cholerae* O139 [54]. The lack of cross-protection between these two serogroups was subsequently demonstrated in animal studies [55]. Although heterologous protection studies have not been performed in volunteers, at least short-term protection against subsequent rechallenge with the homologous O139 strain has been demonstrated in volunteers at the Center for Vaccine Development [56].

The O139 epidemic was caused by a *V. cholerae* strain that is very similar to O1 El Tor strains in numerous phenotypic and genetic traits [54,57]. However, O139 differs from O1 in that a different LPS antigen and a capsular polysaccharide not found in O1 strains is expressed by O139 strains [57]. At the molecular level, the O139 clone appears to have arisen by the substitution of about 22-kb of DNA encoding the O1 LPS with a unique 35-kb region involved in expression of the O139 LPS and capsular polysaccharide [58]. The experience gained from construction of attenuated O1 vaccine candidates provided the basis for the rapid development of live attenuated vaccine candidates for the prevention of cholera due to *V. cholerae* O139.

A. CVD 112

The O139 vaccine candidate CVD 112 was constructed from wild type O139 strain AI1837 by deleting from the *V. cholerae* chromosome the core region that contains the *ctxAB*, *zot*, and *ace* genes [59]. Next,

the gene encoding the cholera toxin B subunit was cloned into the gene encoding the hemolysin of *V. cholerae* (*hlyA*) along with *mer* genes encoding mercury resistance. The *ctxB* was cloned under the control of the *ctx* promoter in an identical manner as in CVD 110. The *ctxB* and *mer* genes were recombined into the chromosome of the AI1837 derivative lacking the core region to yield CVD 112.

CVD 112 was fed to volunteers in doses of 10^6 and 10^8 CFU with buffer [59]. At the lower dose, no adverse reactions such as diarrhea, headache, nausea, fever, or abdominal cramps occurred among six recipients. At the higher dose, 3 of 6 volunteers passed a few small-volume diarrheal stools without any accompanying symptoms. Five weeks after vaccination, 8 vaccin es and 15 unvaccinated control subjects were challenged with wild-type O139 strain AI1837. Diarrhea occurred in 1 vaccinee and 12 control subjects after challenge. The vaccine efficacy of 84% was remarkably similar to protection conferred by primary wild-type infection with AI1837 against subsequent challenge (80%) [56].

In contrast to O1 infection, vibriocidal antibodies against the O139 strain were not associated with protection against rechallenge after either a primary wild-type infection or CVD 112 vaccine [56,60]. Of the 8 (47%) of 17 volunteers who generated vibriocidal antibody following primary infection, 7 (88%) of 8 were fully protected from clinical disease following rechallenge. However, 7 of 9 (78%) of volunteers who had no vibriocidal antibody after vaccination with AI1837 or CVD 112 were also fully protected against rechall nge. The kinetics and magnitude of the vibriocidal response after O139 infection differ from those after O1 infection [60,61]. The methods used to detect O139 vibriocidal antibodies differ slightly from those used to detect O1 vibriocidal responses, and titers may differ depending on the O139 strain used in the assay and the presence or absence of polysaccharide capsule in the target strain [37,60].

B. Bengal 15

Another promising attenuated O139 vaccine candidate was constructed from the wild-type O139 strain MO10. As with CVD 112, the core element containing *ctxA*, *ace*, *zot*, and *cep* genes was deleted along with the flanking RS1 and attRS1 sequences. The *ctxB* gene was cloned under the control of the *htpG* heat-shock promoter and inserted into the chromosomal *recA* gene to give rise to the vaccine candidate Bengal-3 [48]. A spontaneous, nonmotile derivative of Bengal-3 was isolated that lacked flagella and was designated Bengal-15 [62]. In initial clinical trials, 1 of 4 volunteers who received 10^7 CFU of Bengal-3 and none of 10 recipients of 6×10^6 or 10^8 CFU of Bengal-15 experienced diarrhea upon vaccination [62]. In a challenge study, diarrhea occurred in 1 of 7 Bengal-15 vaccinees and 6 of 7 unimmunized controls, for a vaccine efficacy of 83% [62].

C. Carrier-Based O139 Vaccine

A different approach to attenuated live vaccine against O139 *V. cholerae* was recently reported by Favre et al. [63]. Rather than attenuate a wild-type O139 strain, these authors used the O1 vaccine strain CVD 103-HgR as a carrier of O139 antigens. These investigators cloned genes encoding the short core-linked O polysaccharide (SOPS) and the highly polymerized capsular polysaccharide (CPS). A derivative of CVD 103-HgR was constructed that lacked *rfbAB* genes necessary for expression of the O1 LPS. The cloned genes encoding the O139 polysaccharides were then incorporated into the chromosome of the CVD 103-HgR derivative. The resulting strain, CH25, expressed both O139 SOPS and CPS and stimulated high levels of anti-SOPS and anti-CPS serum antibodies when injected intramuscularly into rabbits. No clinical trials have yet been reported with this vaccine candidate, but if clinical results are promising, the extensive safety record accumulated for CVD 103-HgR and the fact that it has already been licensed by regulatory agencies in several countries should speed licensing of such a hybrid vaccine.

VI. POTENTIAL SAFETY ISSUES OF LIVE ORAL CHOLERA VACCINES

As with attenuated live vaccines for other diseases, there is a potential safety issue for live cholera vaccines that is not present for killed or subunit vaccines. Namely, viable bacterial or viral vaccine strains can revert to virulence and actually cause disease. Moreover, live vaccines can be shed and transmitted to nontarget contacts. The rare cases of type 3 polio caused by the live oral Sabin polio vaccine demonstrate this potential danger for any vaccine that is attenuated by mutation of one or a few nucleotides. However, recombinant attenuated cholera vaccines do not have this potential problem because the mutations in the vaccine strains involve the deletion of hundreds of nucleotides. In CVD 103-HgR, the deletion of 540 bp in the *ctxA* gene means that 94% of the mature A_1 protein, the enzymatically active subunit of cholera toxin, has been eliminated [12]. Such a mutation cannot be overcome

by the reversion of one or two nucleotides. Such large deletion mutations provide an unprecedented margin of safety for attenuated vaccines.

It has been suggested that an attenuated cholera vaccine strain could reacquire *ctxA* genes from a wild-type *V. cholerae* strain either in the intestine or in the environment [47]. Even if such a transfer of *ctx* into CVD 103-HgR were to occur, there would be minimal if any adverse consequences. For such a transfer to occur, there must already be large numbers of toxigenic *V. cholerae* present in the intestine, representing simultaneous co-infection, or toxigenic *V. cholerae* pres nt in the environment to serve as the source of *ctx* genes. The impact of one more toxigenic strain of *V. cholerae* in an environment where a single patient can excrete up to 10 L of watery stool containing 10^7–10^8 toxigenic *V. cholerae* per milliliter per day would be insignificant. Like all pathogenic organisms, *V. cholerae* can indeed evolve and acquire new attributes; the emergence of the O139 serogroup is evidence of this capacity. However, rare theoretical possibilities with minimal practical consequence—such as reacquisition of *ctx* genes by attenuated cholera vaccines—are not significant compared with the normal evoluton of wild-type pathogens.

Nevertheless efforts have been made to diminish the possibility of this event. As discussed above, Peru-15, the attenuated El Tor vaccine candidate developed by Mekalanos and colleagues [19,20], was constructed to contain a *recA* mutation and deletion of the RS1 and attRS1 sequences to prevent this transfer. Waldor and Mekalanos [64] have recently shown that *ctx* genes are encoded on a lysogenic bacteriophage and that this phage can mediate transfer of *ctx* between *V. cholerae* strains in vitro and in vivo in mouse intestines even when the *recA*, RS1, and attRS1 sequences are mutated. Interestingly, the receptor for the phage is the TCP pilus, an essential intestinal colonization factor. Mutation of the *tcpA* gene prevents bacteriophage-mediated gene transfer, the only mutation so far known to prevent this transfer [64]. Howev r, attenuated *V. cholerae* O1 strains mutated in the *tcpA* gene do not stimulate an immune response and provide no protection against challenge in volunteer studies [22]. Thus, engineering *V. cholerae* to prevent a theoretical re-acquisition of *ctxA* genes by an attenuated vaccine strain comes at a very high cost—namely, vaccine efficacy.

When CVD 103-HgR was tested for reacquisition of *ctx* genes in vitro and in mice where conditions encourage expression of the phage receptor, no transfer was seen [65]. Whether these results indicate a genuine inability of this vaccine strain to serve as a recipient for the bacteriophage is not known. If the potential reacquisition of *ctx* genes in the environment by live cholera vaccine strains is a concern, then CVD 103-HgR provides an additional level of safety compared to other attenuated cholera vaccines because excretion of the organism is minimal. From 0.5–17% of vaccinees in endemic areas shed the vaccine organism in their stools [29,31–33,35,36], and the concentration of *V. cholerae* CVD 103-HgR organisms in the stool has not exceeded 2×10^2/g [24]. The potential for environmental contamination and transfer of genes in a real-life situation was tested during a trial in Indonesia in which Moore swabs placed in sewers and latrines near households of vaccine recipients failed to recover any vaccine organisms [32].

VII. MULTIVALENT LIVE ORAL CHOLERA VACCINE

Although the attenuated live oral cholera vaccine strain CVD 103-HgR has proved safe in large-scale safety trials in a variety of populations and demonstrated protective efficacy against wild-type O1 *V. cholerae* in volunteer challenge studies, it is not capable of protecting against disease due to the O139 serogroup of *V. cholerae*. The need for incorporating O139 antigens in a cholera vaccine will be determined by the changing epidemiology of disease due to O139. When O139 first appeared in Bangladesh and India in 1992, it appeared that this serogroup might completely displace O1 cholera and lead to an eighth pandemic [54]. However, by 1995, O1 had returned and O139 had nearly disappeared [52,53]. Sporadic cases due to O139 have been reported in Nepal, Thailand, China, Malaysia, Burma, and Singapore, but in none of these countries has O139 displaced O1 [54]. Thus it appeared that O139 was only a brief interruption in the otherwise continual dominance of O1. However, in the fall of 1996, O139 returned to Calcutta [52], and at this point, it is impossible to predict whether O139 and O1 will coexist or whether one or the other serogroup will be dominant.

If a multivalent vaccine is needed, then the O1 vaccine strain CVD 103-HgR could be combined with an O139 vaccine candidate such as CVD 112, Bengal 15, or CH25 to provide protection against both serogroups. As noted above, there is some evidence that as yet undefined El Tor antigens may produce better immunity against mild forms of diarrhea caused by El Tor strains than a classical vaccine. Since O139 strains are essentially identical to El Tor O1 strains except for the surface polysaccharide, a combination of CVD 112 (derived from a wild-type O139 strain) and CVD 103-

HgR could provide El Tor antigens, classical antigens, O1 polysaccharide antigens, and O139 polysaccharide antigens in a bivalent vaccine. If such a bivalent vaccine could be improved by the addition of an O1 El Tor strain, then a trivalent vaccine could be employed consisting of the El Tor CVD 111 vaccine or similar El Tor vaccine candidate, CVD 103-HgR, and an O139 vaccine strain. The demonstration of safety and efficacy of these multivalent, single-dose vaccines in well-designed field trials will lead to new public health tools for the prevention of endemic and epidemic cholera.

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New and Improved Vaccines Against Cholera

Part ii: Oral B Subunit Killed Whole-Cell Cholera Vaccine

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I. INTRODUCTION

Diarrheal disease remains as one of the leading global health problems. It has been estimated that 3–5 billion episodes of diarrhea, resulting in approximately 5 million deaths, occur annually in developing countries, with the highest incidence and severity in children below the age of 5. About half of these diarrheas are caused by bacteria that produce one or more enterotoxins. Cholera, resulting from infection with *Vibrio cholerae* bacteria, is the most severe of these "enterotoxic" enteropathies, while infection with enterotoxigenic *Escherichia coli* (ETEC) is causing the largest number of cases [1,2].

Vibrio cholerae of serogroup O1 is the prototype for the enterotoxin-producing bacteria and was first isolated by Robert Koch in 1884. It can appear as either of two main different serotypes, Inaba and Ogawa. Until the beginning of this century all *V. cholerae* O1 isolates were of the same "classic" biotype. In 1906, however, vibrios of a new biotype, El Tor, were isolated, and for many years vibrios of either the classic or El Tor biotype were isolated from cholera cases. During the early part of the nineteenth century, cholera started to spread from its likely ancient home in Bengal, and since then seven large pandemics have been described that have affected large parts of the world. The latest pandemic that took its departure from Celebes in 1961 has spread to and become endemic in many countries in Asia and Africa, and beginning in 1991, cholera has also reappeared as a significant health problem throughout most of South and Central America for the first time in more than 100 years [3]. The causative agent in Latin America appears to be identical to that of the seventh pandemic O1 El Tor organisms isolated from Asia and Africa.

Recently *V. cholerae* of a "new" serogroup, O139, has emerged as an additional cause of cholera in India and Bangladesh [4]. Cholera caused by *V. cholerae* O139 bacteria has since then been reported from a large number of neighboring countries, e.g., Burma, Indonesia, Malaysia, Nepal, Thailand, and Western China. Although as yet restricted to Southeast Asia and in 1995–1996 accounting for only a small and declining percentage of all cholera cases also in this part of the world, it cannot be excluded that *V. cholerae* O139 may follow in the tracks of *V. cholerae* O1, reaching Africa and Latin America.

In endemic areas, the highest incidence of cholera is seen in children below 5 years of age. Nevertheless, approximately two-thirds of all *V. cholerae* O1 cases occur in older children and adults in these areas [1,5]. On the other hand, when cholera has spread to new countries, all age groups have been affected to the same degree. This is probably because the natural immunity that normally develops with increasing age in endemic countries is missing in newly affected populations [6]. Similarly, cholera caused by the new serogroup O139 has been recorded at least as frequently

in adults as in children [4]. The total number of cholera cases annually is uncertain, since several affected countries and/or areas do not monitor and/or report the disease accurately. The recent outbreaks of *V. cholerae* O1 in Latin America as well as of *V. cholerae* O139 in Asia have probably resulted in a substantially increased number of cholera cases in the last 5 years. Therefore, often cited figures of 3–5 million cases and 150,000–200,000 deaths from cholera annually may well be substantial underestimates of the present situation.

The disease caused by the enterotoxin-producing bacteria is characterized by watery stools without blood and mucus [2]. In cholera, which has the highest frequency of dehydrating disease, the most severe cases can purge as much as 15–25 L of water and electrolytes per day and the mortality rate in severe, untreated cholera is 30–50%.

No effective vaccines for use in humans against either cholera or ETEC diarrhea have been available until very recently. Thus, previous parenteral cholera vaccines have induced up to 50% protection for only 3–6 months [7] and vaccines against ETEC and other enterotoxin-producing organisms have been lacking completely. The limitations in protection induced by the previous cholera vaccines could probably be explained mainly by the parenteral administration route used. Thus, in individuals who have not been immunologically primed by previous natural exposure to *V. cholerae*, injectable cholera vaccines give rise to little if any immune responses locally in the gut, i.e., where both the bacteria and the toxin they produce exert their action during infection and where local immunity is of critical importance. Moreover, in the previously "primed" host, the parenteral injection route is much less efficient than oral immunization [8,9].

In recent years, however, an inactivated oral cholera vaccine that in large field trials has been shown to afford 85% protection for the first 6 months and more than 50% protection for 2–3 years has been developed and licensed [10,11]. As described in another chapter of this volume, a live oral cholera vaccine, CVD 103-HgR, has also been developed and found to be safe and immunogenic and to give rise to good protection against challenge with *V. cholerae* O1 in human volunteers [12,13]; the protective efficacy and the duration of protection of the live vaccine against natural disease are presently being tested in a field trial in Indonesia. In this review, we describe the development and testing of oral inactivated vaccines and their ability to protect against cholera. Vaccine formulations comprising cholera toxin B subunit as a component also provide significant short-term protection against ETEC diarrhea.

II. MECHANISMS OF IMMUNITY

The development of the new cholera vaccines has to a large extent been based on the new insights into the mechanisms of disease and immunity in cholera and related enterotoxin-induced diarrheas that have been achieved during the last 25 years [11]. The main findings guiding modern vaccine development may be summarized as follows:

1. The pathogenesis critically depends on both bacterial colonization and toxin production and action locally in the small intestine.
2. Either or both of antibacterial and antitoxic immunity capable of preventing these events can protect against disease.
3. Both antibacterial and antitoxic immunity mainly depend on locally produced antibodies of the secretory IgA (SIgA) type, an these antibodies are preferentially stimulated by oral-enteric (rather than parenteral) vaccination.
4. Antibacterial and antitoxic mucosal immune mechanisms (SIgA antibodies) can cooperate synergistically in protecting against disease.

A. Toxins and Antitoxic Immunity

As stated, the major pathogenic mechanisms of both *V. cholerae* and other enterotoxigenic bacteria include initial bacterial colonization of the small intestine followed by the elaboration of enterotoxin(s) that, through specific mechanisms, can induce electrolyte and water secretion, resulting in diarrhea [11,14,15]. These enterotoxins, which have a cytotonic rather than cytotoxic effect on the intestinal epithelium, are believed to stimulate secretion, primarily from the crypt cells in the upper part of the small intestine, by inducing increased formation of cyclic AMP and/or cyclic GMP in the epithelial cells. Cholera toxin (CT) is the prototype enterotoxin and is produced by most *V. cholerae* O1 bacteria of either the classic or El Tor biotype. Cholera toxin (CT) with identical structure to O1 El Tor CT is also formed by the novel *V. cholerae* O139 serogroup [16]. Cholera toxin consists of five identical binding (B) subunits associated in a ring into which a single toxic-active (A) subunit is noncovalently inserted; the binding receptor for the CT on cells is a specific glycolipid, the ganglioside GM1 [14]. ETEC bacteria may produce either or both of a unrelated heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST) [11,15]. Of these, LT is structurally and immunologically closely related to CT.

Studies in experimental animals have shown a direct correlation between protection against CT-induced

fluid secretion and intestinal synthesis of SIgA antibodies and also between protection and the number of SIgA antitoxin-producing cells in the intestine [11]. A protective role of SIgA antitoxin has also been clearly indicated by the direct correlation in breast-fed children in Bangladesh of a reduced risk to get cholera (ven when infected with *V. cholerae* O1) when SIgA antitoxin is above a certain level in the ingested milk [17]. Furthermore, as discussed below, vaccine-induced production of antitoxic immunity associated with intestinal SIgA antitoxin production has in a large field trial in Bangladesh been shown to confer significant protection against both cholera and LT ETEC diarrhea. The identification of the subunit structure of CT an LT and the roles of the different subunits in pathogenesis and immunity have indicated that the purified cholera or LT B-subunits (CTB or LTB) are suitable toxoid candidates. Antitoxic immunity in both cholera and LT ETEC diarrhea is mainly if not exclusively mediated by locally produced antibodies directed against the B subunit portion of the toxin molecules. Furthermore, the B-subunit pentamer has been found to be particularly well suited as an oral immunogen because it is stable in the intestinal milieu and capable of binding to the intestinal epithelium, including the M cells of the Peyer's patches—properties which are important for stimulating mucosal immunity and local immunological memory [18].

B. Colonization Factors and Antibacterial Immunity

It is well established that *V. cholerae* O1 lipopolysaccharide (LPS) is the predominant antigen affording antibacterial immunity against experimental O1 cholera [8]. Recent studies have suggested that immunity to *V. cholerae* O139 is also to a large extent provided by antibodies to LPS. An important observation guiding the design of new cholera vaccines has concerned the cooperation between antitoxic and antibacterial immune mechanisms in cholera. The main protective antibodies against cholera have been identified as directed against the cell wall LPS and CTB [19]. Either of these two types of antibodies can confer protection against disease by inhibiting bacterial colonization and toxin binding, respectively; when present together in the gut, they can have a strongly synergistic protective effect [20].

In *V. cholerae* O1 bacteria of the classic biotype, a toxin-coregulated pilus (TCP) has been shown to be of importance for colonization of the small intestine [21]. Recent evidence indicates that for *V. cholerae* O1 El Tor and O139, an antigenically distinct form of TCP is also important for colonization and disease [22]. In addition, *V. cholerae* bacteria have been found to express a number of other fimbrial structures—e.g., the mannose-sensitive hemagglutinin (MSHA), which is found on O1 El Tor and O139 *V. cholerae* but not on O1 bacteria of classic biotype—which can mediate bacterial attachment to epithelial cells. The role of these other attachment factors for colonization and infection in humans remains to be defined [23]. The identification of TCP as an important colonization factor on *V. cholerae* suggests that it should be possible to raise protective antibacterial immunity against these fimbrial antigens. Indeed, in experimental systems, it has been found that monoclonal antibodies or polyclonal antisera against TCP can protect against infection and disease [24]. However, following natural or experimental infection, little if any anti-TCP immunity develops [24a]; as an overall conclusion, it remains to be defined whether mucosal immune responses against TCP and other surface antigens on *V. cholerae* can add significantly to the protective action mediated by antibodies to O1 (or O139) LPS antigen.

III. ORAL WHOLE-CELL AND B SUBUNIT WHOLE-CELL O1 CHOLERA VACCINES

An oral cholera vaccine consisting of the nontoxic, highly immunogenic CTB protein in combination with heat- and formalin-killed *V. cholerae* O1 classic and El Tor vibrios (Table 1) has been developed and is now

Table 1 Composition of Oral B Subunit Whole-Cell (B-WC) Cholera Vaccines—Composition per Dose

A. B-O1 WC (Bangladesh field trial formulation)
1 mg CTB (purified from CT) + 1×10^{11} killed bacteria
- 2.5×10^{10} heat-killed Inaba vibrios (strain Cairo 48)
- 2.5×10^{10} heat-killed Ogawa vibrios (strain Cairo 50)
- 2.5×10^{10} formalin-killed classic vibrios (strain Cairo 50)
- 2.5×10^{10} formalin-killed El Tor vibrios (strain Phil 6973)

B. rB-O1 WC (Currently licensed formulation)
1 mg recombinant CTB + same WC composition as in A

C. Bivalent rB-O1/O139 WC
Same rB-O1 WC as in B + 5×10^{10} formalin-killed O139 vibrios (strain 4260B grown and formalin-inactivated to express fimbrial antigens such as MSHA)

a licensed vaccine [11]. This CTB whole-cell (B-WC) vaccine, which is given together with a bicarbonate buffer to preserve the CTB pentameric structure, has in extensive clinical trials, including large field trials, proved to be completely safe and to provide good protection against cholera and also partial protection against diarrhea caused by LT-producing ETEC.

A. Protection Against Cholera

The B-WC vaccine was designed to evoke antitoxic as well as antibacterial intestinal immunity, since in animal studies these types of immunity have been shown to provide synergistic cooperative protection [8,20]. Phase I and II clinical studies established that the B-WC vaccine does not cause any detectable side effects and that, after either two or three doses, it stimulates a gut mucosal IgA antitoxic and antibacterial immune response (including memory) comparable to that induced by cholera disease itself [9,25,26]. Furthermore, immunization with the complete B-WC vaccine was found to protect American volunteers against challenge with a dose of live cholera vibrios (biotype El Tor) that caused disease in 100% of concurrently tested unvaccinated controls. In a follow-up study, it was shown that also oral vaccination with the WC component alone induced protection against challenge, which was only marginally less than that afforded by the B-WC vaccine [27].

On this basis, a large, double-blind, placebo-controlled field trial with more than 90,000 participants was undertaken in rural Bangladesh. The results established that both the B-WC vaccine and the WC component alone confer long-lasting protection against cholera. The B-WC vaccine had a higher initial efficacy level than the WC vaccine (85 versus 58% for the initial 4- to 6-month period) in support of the significant protective immunogenicity of the CTB component [28]. Indeed, if for calculation of the protective efficacy of the CTB component one estimates the protective efficacy of B-WC in comparison with WC by looking at the WC group as "placebo," the protective efficacy of the B subunit was 73% during this period. The B-WC continued to be significantly more protective than the WC-alone vaccine for the first 8 months after vaccination. Thereafter, however, for a 3-year follow-up period, the efficacy was similar, approximately 50% for both vaccines [10]. Still higher (about 70%) long-term protective efficacy was seen in those over age 5 when vaccinated. These figures correspond to an estimated overall protective efficacy of ca 60% in the population aged 2 years and above assuming that adult males who were now excluded from analyses would have been protected to the same extent as adult females. Protection was of similar magnitude after 2 or 3 doses of vaccine [10]. It seems likely that the age group below 5 years, in which immunity largely waned after the initial high-level protection for the first 6–9 months, could also be provided with long-lasting high-level protection by a booster immunization after 1 year.

In the initial vaccine formulation tested in Bangladesh, the CTB component, was prepared by chemical isolation from CT produced by the high-expression wild-type strain 569B [29], which made the preparation of this component relatively laborious and expensive. It was therefore a significant improvement when Sanchez and Holmgren [30] constructed an efficient recombinant overexpression system for the large-scale production of CTB. Based on this, it has since been possible to further increase and simplify the production and downstream purification of recombinant CTB for industrial vaccine production purposes [J. Holmgren and SBL Vaccin, unpublished data]. Extensive clinical testing of a second-generation vaccine formulation based on such recombinantly produced CTB (rCTB) has in different settings shown the same degree of safety and immunogenicity as the Bangladesh trial formulation; therefore this has become the currently produced and licensed vaccine formulation. A recent field trial in Peru has also confirmed the strong protective efficacy of the rB-WC formulation. Thus, Sanchez et al. [31] found that this vaccine, given in two doses together with a bicarbonate buffer, conferred 86% protection against cholera in Peruvian military recruits. It is especially noteworthy that this high level of protection, being very similar to the 85% protection seen for the first 6-month postvaccination period in Bangladesh, in the Peruvian setting was (1) obtained with two doses of vaccine given only 1-2 weeks apart; (2) directed against severe cholera of exclusively the O1 El Tor biotype, which is usually more difficult to protect against than against classic biotype cholera; and (3) achieved in a population being almost exclusively of blood group O. These factors that earlier had been thought by some to possibly reduce the efficacy of the vaccine as compared with the findings in the Bangladesh trial.

In Vietnam, a locally produced inexpensive WC vaccine similar to the Swedish WC vaccine was recently evaluated in a large-scale field trial conducted in more than 22,000 households in the central coastal city of Hue. Persons aged over 1 year were allocated in alternate households to receive two doses of vaccine or no vaccine (67,000 persons in each group). During an outbreak of El Tor cholera that occurred 8–10 months after vaccination, 66% protection was noted among the persons who received the two-dose vaccine

regimen; protection was similar for children age 1–5 years (68%) and for older persons (66%) [33]. These findings clearly lend encouragement to the notion that an inexpensive, locally produced, and effective oral cholera vaccine may be within reach of the limited health care budget of poor countries with endemic cholera [33].

B. Protection Against ETEC Diarrhea

Through its B-subunit component, the B-WC vaccine also has been shown to provide substantial short-term protection against diarrhea caused by ETEC [34,35]. ETEC is, together with rotavirus, the most common cause of diarrhea in children in developing countries in Asia, Africa, and Latin America. ETEC also continues to be a common cause of diarrhea in adults [1]. Although it has been estimated that only about one-third of all ETEC infections are symptomatic in children in endemic areas, this is enough to result in at least 650 million episodes of diarrhea and about 800,000 deaths annually in children below the age of 5. In addition, ETEC is without comparison the most common cause of traveler's diarrhea [36]. Indeed, it has been estimated that approximately 50% of persons traveling to developing countries experience diarrheal disease, and ETEC is isolated in one-third to half of these episodes.

In the cholera vaccine trial in Bangladesh, it was found that the oral B-WC cholera vaccine, specifically through its CTB component, afforded significant protection against diarrhea caused by LT-producing ETEC [34]. The protection observed was about 67% for 3 months, and, interestingly enough, was equally strong against bacteria producing LT alone as against bacteria producing LT in combination with ST. The protection was more pronounced against ETEC diarrhea associated with severe, life-threatening dehydration, which was reduced by 86% during the first few months after immunization, than against milder disease (56% efficacy).

Another prospective double-blind study was conducted among tourists who went to Morocco from Finland. A total of 307 tourists received two oral doses of B-WC cholera vaccine, whereas 308 controls received two doses of a placebo (killed *E. coli* K12 bacteria) before departure. Clinical and microbiological evaluation of diarrhea in vaccinees and controls showed that the vaccine had a highly significant protective efficacy against LT-producing *E. coli*, whether isolated as the single enteric pathogen or combined with *Salmonella* or other enteric pathogens. Thus, protection against LT-producing ETEC (either LT only or LT/ST) was 60%, and the vaccine conferred 65% protection against diarrhea caused by ETEC in combination with any other pathogen isolated and no less than 82% protection against diarrhea associated with isolation of the combination of ETEC and *Salmonella* [35].

C. Public Health Aspects

In addition to their disease-specific efficacy in the Bangladesh field trial, both the B-WC and the WC vaccines substantially reduced the overall diarrhea morbidity among those vaccinated, such that there was a 50% reduction in admissions for life-threatening diarrhea in the vaccinated as compared with the placebo group over each of 3 years of follow-up [37] (J. Clemens, unpublished data). The latter finding provides ample evidence of the public health application potential of the B-WC cholera vaccine in settings such as are being found not only in Bangladesh but also in many other countries, where cholera, ETEC, and other enterotoxic diarrheal diseases account for a large number of life-threatening watery diarrheas and where adequate treatment facilities in rural areas often are scarce [33,38].

In the first year of follow-up after vaccination in Bangladesh, there was a dramatic effect of both the B-WC and WC vaccines on total mortality as compared with placebo. Thus, overall mortality rates were 26% lower in the B-WC group and 23% lower in the WC group during the first year. Among women vaccinated at ages >15 years, those who received B-WC had 45% fewer deaths ($p < 0.01$) and those who received WC had 33% fewer deaths ($p < 0.05$) than placebo recipients. Several additional findings suggested that this reduction in overall mortality was a specific effect rather than a statistical coincidence: (1) the effect was restricted to the high-cholera season, (2) it was correlated with deaths associated with or preceded by diarrheal disease according to "verbal autopsy" reports by household members, and (3) as mentioned, it was limited to the underprivileged group of women rather than children participating in the study [37]. However, differing from the observation mentioned above that vaccination significantly reduced the incidence of life-threatening watery diarrheas in both adult women and children over each of the 3 follow-up study years, the effect on total mortality was restricted to the first year. It therefore remains to be corroborated whether indeed even in a "well-treated" area such as the field site in Matlab there is a significant number of hidden cholera and severe ETEC diarrhea deaths that might be averted by effective cholera and/or ETEC vaccination programs.

One special aspect studied in the Bangladesh field trial of oral cholera vaccines was the association be-

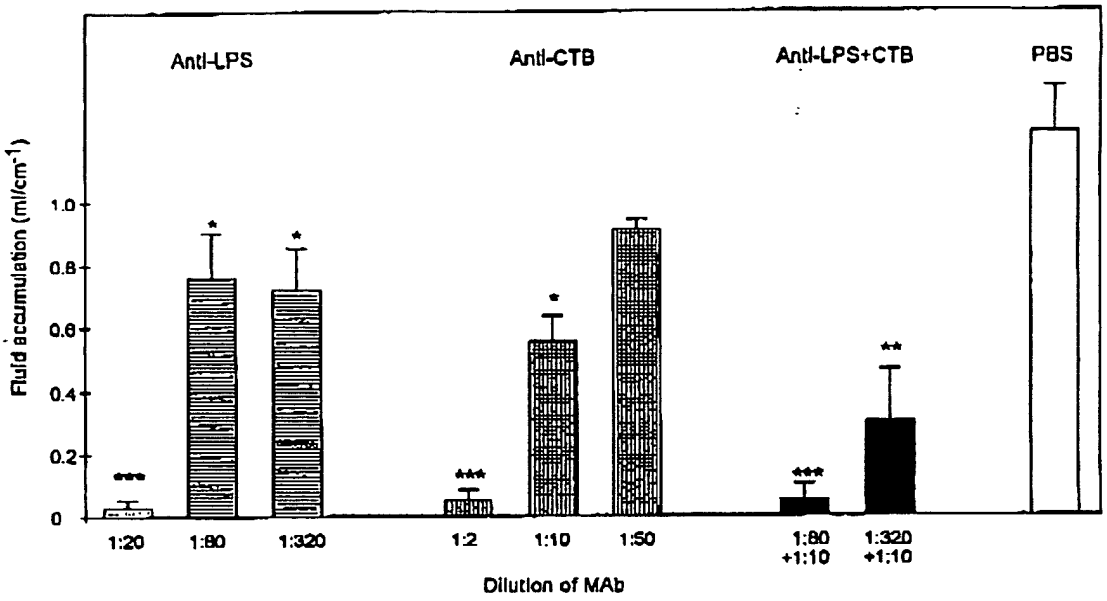

Figure 1 Synergistic protective effect of MAbs against O139 LPS (MAbs O139:1:1:9 and O139:20:4:5 combined 1:1) and CTB against fluid accumulation in rabbit ileal loops caused by challenge with live *V. cholerae* O139 strain 4260B. Results show mean values (+SEM) of eight experiments. Challenge dose, 2×10^6 bacteria (4 ED_{50}). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus PBS control.

tween breast-feeding and the risk of severe cholera among children under 36 months of age [39]. The results showed that overall, breast-feeding was associated with a 70% reduction of the risk of developing clinical severe cholera, and exclusive breast feeding during infancy appeared to be associated with nearly 100% protection against severe cholera. Furthermore, maternal vaccination with either of B-WC or WC oral cholera vaccines was associated with a 50% reduced risk of their non-vaccinated children to develop severe cholera. These results raised a possibility that vaccination of mothers may provide protection to their young children in endemic settings by either or both of interrupting maternal-child transmission of cholera and increasing the specific immune protecting potential of the mother's breast milk.

IV. COMBINED B-WC VACCINE AGAINST O1 AND O139 CHOLERA

Based on the emerging significance since late 1992 of *V. cholerae* O139 as an additional cause of epidemic cholera in Southeast Asia, much recent attention has been focused on the possibility to develop a cholera vaccine that also affords protection against this "new" type of cholera. We have therefore studied the immune mechanisms and protective antigens of *V. cholerae* O139 in animal models as a basis for vaccine development and then in collaboration with SBL Vaccin, Sweden (P. Askelöf, U. Bjare and H. Wigzell), we have developed an oral bivalent B subunit-O1/O139 whole-cell cholera vaccine, which is now in clinical testing.

Based on a broad characterization of different clinical isolates of *V. cholerae* O139 Bengal with regard to properties deemed to be relevant for vaccine development, we selected one typical strain, 4260B, as a candidate inactivated vaccine strain. This strain—having a well-exposed O antigen as well as capsule and the capacity to produce large amounts of CT and mannose-sensitive hemagglutinin (MSHA) pili but minimal production of the proteolytic soluble hemagglutinin—was used as immunogen for production of antibacterial antisera. Antisera against live or killed O139 vibrios (4260B) conferred passive protection against fluid accumulation in rabbit intestinal loops induced by challenge with the homologous as well with heterologous O139 strains (Figure 1). The protective effect of antisera was correlated to the anti-LPS antibody titers rather than to titers against whole bacteria that had been grown for TCP expression and

Table 2 Intestinal Antitoxin and Antibacterial IgA Antibody Responses After Two or Three Oral Immunizations with B-O1/O139 WC Cholera Vaccine

	Frequency (and magnitude)[a] of responses			
	Lavage		Feces	
Immune response to	Maximal[b] (n = 9)	Two doses (n = 9)	Maximal (n = 10)	Two doses (n = 10)
CTB	100% (16.5)	100% (11.8)	90% (13.4)	90% (9.6)
O1 vibrios	78% (2.8)	78% (2.8)	70% (8.9)	70% (4.7)
O139 vibrios	78% (2.8)	78% (2.7)	80% (7.4)	70% (4.7)

[a]Percent vaccinees responding with a significant, i.e., ≥twofold increase in specific IgA antibody titer/total IgA between pre- and postimmunization samples; geometric mean magnitudes of antibody responses among responders are given within brackets.
[b]Maximal immune responses after two or three immunizations with B-O1/O139 WC cholera vaccine.

was substantially higher than the protection conferred by antisera to CT or CTB. Antitoxic immunity by itself appeared to be less significant against O139 challenge than against O1 challenge and thus also less important than anti-O139 (anti-LPS) antibacterial immunity. However, monoclonal antibodies to O139 LPS and CTB/CT exhibited a strong synergistic protection against O139 challenge irrespective of the level of sensitivity of challenge strains to monoclonal antibodies against O139 LPS in vibriocidal assays in vitro [40] (Figure 1).

Based on these findings, we have, together with SBL Vaccin (Stockholm), developed an oral bivalent B subunit-O1/O139 whole-cell (B-O1/O139 WC) cholera vaccine by adding formalin-killed O139 vibrios of strain 4260B to the recently licensed oral rB-O1 WC vaccine (Table 1). When tested in Swedish volunteers, this rB-O1/O139 WC vaccine was found to be safe and immunogenic [41]. Two vaccine doses given 2 weeks apart induced strong intestinal-mucosal IgA antibody responses to CT (100%), O1 vibrios (78%), and O139 vibrios (78%) as tested by enzyme-linked immunosorbent assays (ELISAs) using pre- and postvaccination intestinal lavage or fecal extract specimens (Table 2). These gut IgA antibody responses were associated with intestine-derived antibody-secreting cell (ASC) responses in peripheral blood (Table 3). A third dose of vaccine given after 5–6 weeks did not result in any further increased immune response. Most volunteers also developed IgA and IgG antitoxin as well as vibriocidal antibody responses in serum that were comparable to those induced by the B-O1 WC vaccine (Table 4). Thus, the O139 component of the vaccine seemed to have a capacity similar to that of the O1 component to induce intestinal and systemic antibacterial immune responses, and its addition to the vaccine did not interfere with the immunogenicity of the B subunit or O1 WC components.

Table 3 Vaccine-Specific Intestine-Derived ASC Responses in Peripheral Blood of Swedish Volunteers After Two or Three Oral Immunizations with B-O1/O139 WC Cholera Vaccine

	Frequency[a] (and magnitude)[b] of responses	
ASC response to	Maximal[c] (n = 12)	Two doses (n = 12)
CTB	100% (457)	100% (427)
O1 vibrios	92% (13.2)	75% (11.1)
O139 vibrios	75% (11.5)	67% (13.1)

[a]Percent vaccinees responding with a significant, i.e., twofold or greater increase in vaccine-specific ASCs between pre- and post vaccination specimens.
[b]Geometric mean fold-increase in ASCs among responders in relation to preimmune ASCs.
[c]Maximal ASC responses after two or three doses of B-O1/O139 WC cholera vaccine.

V. SUMMARY

During the last decade there has been rapid progress in the development of new, much improved vaccines against cholera. These vaccines, which are given orally to stimulate specifically secretory IgA formation and immunologic memory in the gut mucosal immune system, are based on either a combination of purified cholera B subunit (CTB) and killed *V. cholerae* O1 vibrios of the different serotypes and biotypes (B-WC vaccine) or on live attenuated mutant strains of *V. chol-*

Tabl 4 Serum Antibody Responses in Swedish Volunteers After Oral Immunization with Bivalent B-O1/O139 WC Cholera Vaccine or Monovalent B-O1 WC Cholera Vaccine

	Frequency[a] (and magnitude)[b] of responses		
	B-O1/O139 WC vaccine		B-O1 WC vaccine
Immune response	Maximal[c] (n = 12)	Two doses (n = 12)	Two doses (n = 17)
IgA antitoxin	100% (22.4)	100% (22.4)	88% (16.7)
IgG antitoxin	100% (8.7)	100% (7.1)	76% (10.2)
O1 vibriocidal	83% (16.6)	83% (15.8)	65% (16.2)
O139 vibriocidal	67% (21.4)	58% (21.7)	N.T.[d]

[a]Percent vaccinees responding with a significant, i.e., twofold or greater increase in antibody titer between pre- and postvaccination specimens.
[b]Geometric mean maximal titer increase for responders in relation to pre-immune titers.
[c]Maximal antibody responses after two or three doses of B-O1/O139 WC cholera vaccine.
[d]N.T. = not tested.

erae producing CTB (e.g., CVD 103-HgR). The most extensively tested of these new vaccines, the oral B-WC cholera vaccine, has proved to be completely safe. Its excellent immunogenicity associated with high-level short-term protective efficacy (85% for the first 6 months in both children and adults) as well as good long-term protection (~65% over 3 years in vaccinees more than 5 years of age) against cholera have been documented, e.g., in a large, randomized, placebo-controlled field trial in 90,000 persons living in a cholera-endemic area. The newly emerging cholera endemic caused by a new serogroup of *V. cholerae*, O139, has also led us to develop a second-generation CTB-WC cholera vaccine containing the new serotype as an additional WC vaccine component. Because of the cross-reacting enterotoxins the B-WC cholera vaccine also confers significant (60–70%) short-term protection against diarrhea caused by LT-producing enterotoxigenic *E. coli* (ETEC). The introduction of recombinant DNA technology for production of the B-subunit component has facilitated inexpensive large-scale manufacturing of both the cholera and ETEC vaccines. In addition to being useful prophylactic agents in travelers, these vaccines will hopefully become useful public health tools in future control strategies in developing countries.

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